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The synthesis of peptidylfluoromethanes and their properties as inhibitors of serine proteinases and cysteine proteinases

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A synthesis of peptidylfluoromethanes is described that utilizes the conversion of phthaloyl amino acids into their fluoromethane derivatives. These can be deblocked and elongated. The inactivation of chymotrypsin by Cbz-Phe-CH_2F (benzyloxycarbonylphenylalanylfluoromethane) was found to be considerably slower than that of the analogous chloromethane. The fluoromethane analogue inactivates chymotrypsin with an overall rate constant that is 2% of that observed for the inactivation of the enzyme with the chloromethane. However, the result is the same. The reagent complexes in a substrate-like manner, with $K_i = 1.4 \times 10^{-4}$ M, and alkylates the active-centre histidine residue. Cbz-Phe-Phe-CH_2F and Cbz-Phe-Ala-CH_2F were investigated as inactivators of the cysteine proteinase cathepsin B. The difference in reactivity between fluoromethyl ketones and chloromethyl ketones is less pronounced in the case of the cysteine proteinase than for the serine proteinase. Covalent bond formation takes place in this case also, as demonstrated by the use of a radiolabelled reagent.

INTRODUCTION

Since their introduction for the inactivation of chymotrypsin by affinity labelling (Schoellmann & Shaw, 1962; Ong *et al.*, 1965) peptidyl-bromomethanes and -chloromethanes have been extensively studied. Variation of the amino acid sequence in the peptidyl portion provides affinity to individual serine proteinases whose specificities are satisfied, leading to the formation of a reversible complex and subsequent formation of a covalent bond. In serine proteinases this takes place at the active-centre histidine residue, residue 57 in the chymotrypsin sequence, and at the homologous histidine residue in trypsin, thrombin and plasmin as determined by structural analysis. This site of reaction is assumed to be general for other members of this family of proteinases. Cysteine proteinases are also irreversibly alkylated, but at the essential cysteine thiol group (Bender & Brubacher, 1966).

Because variations in the peptide structure have led to selective and irreversible inactivators of proteinases of physiological importance as in blood coagulation (Kettner & Shaw, 1981) and elastolytic degradation (Powers *et al.*, 1977), there has been hope that the variety of useful applications *in vitro* might lead also to applications *in vivo*. Only a few attempts have been made. The observation of some toxic effects on the repeated administration of one peptidylchloromethane (Ranga *et al.*, 1981) may or may not reflect a general difficulty with this type of inhibitor. However, the reactivity towards thiol groups, whether in the form of GSH (Rossman *et al.*, 1974) or in enzymes not related to proteolysis (Pong *et al.*, 1975), is probably an undesirable property, leading to multiple potentially harmful effects. Since the reactivity of chloromethyl ketones towards the active-centre histidine residue of serine proteinases is enhanced by at least 10^6-fold by the proximity effect due to inhibitor–enzyme-complex formation (Shaw & Ruscica, 1971), it seemed attractive to examine fluoromethyl ketones in the hope that the greatly diminished nucleophilic displaceability of fluoride from such compounds might be overcome in enzyme complexes by proximity effects. However, side reactions with other molecules might be diminished owing to negligible rates, and the overall result would be an increase in selectivity. If the fluoride in such derivatives proved to be resistant to displacement even within an enzyme complex, conceivably the derivative could nevertheless be a useful proteinase inhibitor as the result of formation of a stable tetrahedral adduct of the active-centre serine hydroxy group to the carbonyl group of the reagent. This structural arrangement is found in the inhibited enzyme formed from trypsin and a substrate-like chloromethyl ketone (Malthouse *et al.*, 1983), but may not depend on a displacement reaction, as shown by the work of Brodbeck *et al.* (1979) on the inhibition of acetylcholinesterase by fluorinated ketone substrate analogues.

Many attempts to synthesize peptidylfluoromethanes by displacement of chloride or bromide from peptidylhalomethanes by various forms of fluoride failed. Direct synthesis from a diazomethyl ketone by reaction with HF analogous to the rapid formation of bromomethyl or chloromethyl ketones (Schoellmann & Shaw, 1962; Kettner & Shaw, 1981) did not lead to fluorine-containing products. In the case of Cbz-Phe-CHN_2, the use of HF/pyridine, a convenient source of HF (Olah *et al.*, 1973), provided mild conditions in which deblocking could be avoided, and permitted the isolation of a product for which an internally cyclized derivative involving the amide proton is the most likely structure. This reaction is known to occur upon treatment of diazomethyl ketones with acid (Smith & Dieter, 1981),

Abbreviation used: Cbz, benzyloxycarbonyl.
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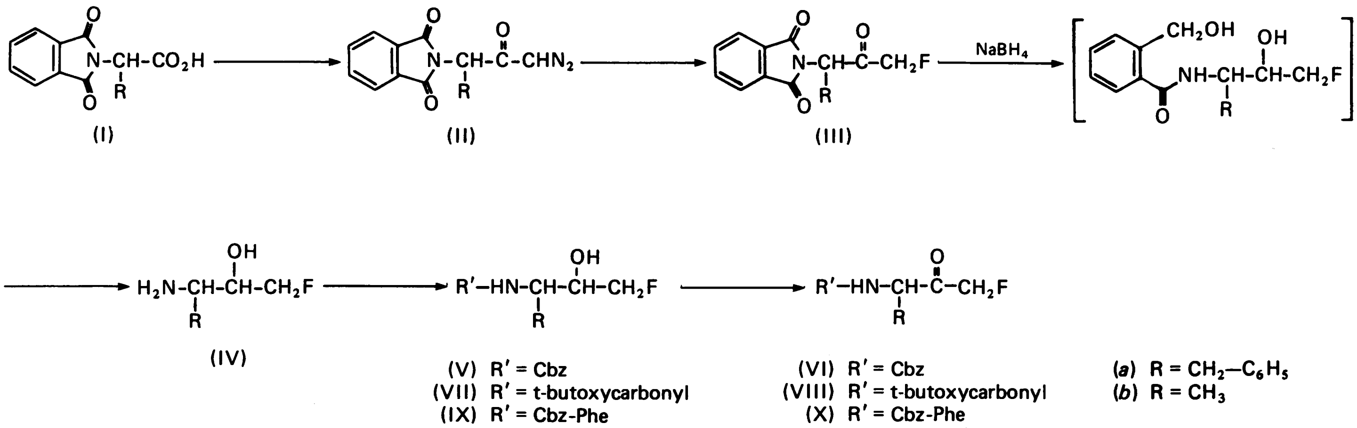

Scheme 1. Synthesis of peptidylfluoromethanes

indicating that the reaction of the intermediate diazonium compound with fluoride is slow relative to the cyclization. The use of phthaloyl-Phe, instead of Cbz-Phe, to remove the amide proton and block the cyclization reaction suggested itself as a promising alternative. The possibility of eventually removing the phthaloyl group under mild conditions (Osby *et al.*, 1984) for elongation of the peptide portion of the structure improved the attractiveness of this approach, which eventually provided a number of peptidylfluoromethanes for enzymic evaluation (see Scheme 1). While this work was in progress, Rasnick (1985) described a different chemical synthesis for this type of peptide derivative and initial results with the inhibition of a thiol proteinase, cathepsin B, and more recently yet another synthetic approach has been published (Imperiali & Abeles, 1986).

EXPERIMENTAL

Materials

Phthaloyl-Phe and phthaloyl-Ala were from Senn Chemicals, chromogenic and fluorogenic substrates were from Bachem, and tritiated acetic anhydride was from New England Nuclear. Cbz-Phe-Phe-CH_2Cl and Cbz-Phe-Ala-CH_2Cl were synthesized from the diazomethanes described earlier (Leary *et al.*, 1977; Watanabe *et al.*, 1979). All other chemicals were from Fluka. Chymotrypsin (EC 3.4.21.1) (bovine, 3 × crystallized) from Sigma Chemical Co. was gel-filtered in 1 mM-HCl and freeze-dried. Cathepsin B (EC 3.4.22.1) was prepared from fresh pig liver as described by Evans & Shaw (1983).

Methods

Inhibition studies with chymotrypsin. To determine the K_i of Cbz-Phe-CH_2F, various amounts of an ethanolic 0.01 M solution of the inhibitor were added to a volume of 0.2 M-Tris/HCl buffer, pH 7.8, containing 0.1 M-NaCl and 0.01 M-$CaCl_2$ such that a final volume of 1.0 ml was achieved after inclusion of substrate. This was 5 mM-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (50 or 20 μl in the same buffer, depending on the final concentration desired). The reaction was started by addition of 5 μM-chymotrypsin (10 μl) and the initial velocity was observed at 410 nm in a Shimadzu recording spectrophotometer set with full scale at 0.25 *A* unit.

Inactivations of a time-dependent nature were measured at 25 or 37 °C in 0.1 M-Pipes/NaOH buffer, pH 7.0. At the lower temperature, a reaction with a final volume of 1.0 ml was prepared in this buffer containing 0.2 ml of a 1 μM solution of chymotrypsin and various amounts of inhibitor. Ethanol, if needed, was added to give a final concentration of 5% (v/v). Samples (50 μl) were assayed at 410 nm with the nitroanilide substrate (50 μl of 5 mM) made up to a final volume of 1.0 ml with 0.2 M-Tris/HCl buffer, pH 7.8. The spectrophotometer was set at 0.05 *A* unit full scale. For inhibitions at 37 °C, residual activity was measured with Ala-Ala-Phe-7-amino-4-methylcoumarin at a final concentration of 0.4 mM in 0.1 M-Pipes/NaOH buffer, pH 7.0, and a recording fluorimeter essentially as described below for cathepsin B.

Inactivation studies with cathepsin B. A solution of the inhibitor under study in dimethyl sulphoxide was added to a solution of cathepsin B (about 0.01 μM) in 0.1 M-sodium acetate buffer, pH 5.4, containing 1 mM-EDTA and 0.02% 2-mercaptoethanol such that the final concentration of inhibitor was 0.1–7.5 μM. The enzyme and inhibitor were incubated at 37 °C, and samples (10–20 μl) were removed and assayed for residual enzyme activity with a solution (2 ml) of the fluorogenic substrate Cbz-Phe-Arg-7-amino-4-methylcoumarin (20 μM) in the above buffer. The dilution involved in setting up the assay effectively stopped further reaction with the inhibitor. The rate of hydrolysis of substrate was determined by measuring the rate of increase of fluorescence at 455 nm (excitation at 383 nm), the concentration of product being obtained by reference to a 1 μM solution of 7-amino-4-methylcoumarin.

Amino acid analysis of chymotrypsin inactivated with Cbz-Phe-CH_2F. Chymotrypsin (2.5 mg) in 0.1 M-Pipes/NaOH buffer, pH 7.0 (4.5 ml), was treated with Cbz-Phe-CH_2F at a final concentration of 0.5 mM. The reaction mixture contained 10% (v/v) ethanol. Samples were assayed fluorimetrically. When the activity had fallen by 98%, the solution was adjusted to pH 3 with 1 M-HCl, gel-filtered through Sephadex G-25 (3 cm × 21 cm) with elution by 1 mM-HCl and the protein peak was freeze-dried. The amino acid composition was determined by standard methods (Tong & Elzinga, 1983). We are grateful to Dr. Marshall Elzinga (Brookhaven

National Laboratory, Upton, NY, U.S.A.) for these determinations.

Radiolabelled enzyme derivatives. Chymotrypsin (2.5 mg) in 0.1 M-Pipes/NaOH buffer, pH 7.0 (20 ml), was left at room temperature with ethanolic 10 mM-[^{3}H]acetyl-Phe-CH_2F (0.8 ml) for 16 h. Activity fell to 13%. The solution was dialysed with four changes of 1 mM-HCl (4 litres) over a 48 h period. The sac contents were freeze-dried and the residue was taken up in 6 M-guanidinium chloride (0.4 ml). After 1 h, the solution was applied to a column (90 cm × 1.7 cm) of Sephadex G-75 equilibrated with 6M-guanidinium chloride and eluted with the same solvent at a flow rate of about 10 ml/h. Fractions (1.3 ml) were collected and the radioactivities of samples (0.2 ml) were counted by scintillation counting.

In the case of cathepsin B, 0.4 ml of a 7.6 μM solution of enzyme was treated with 20 μl of 10 mM inhibitor, which provided a final concentration of 0.48 mM in 5% (v/v) ethanol. Samples (2 μl) were assayed. There was a 68% loss of activity in 90 min with 90% loss upon standing overnight at 4 °C. The reaction was dialysed against 4-litre volumes of water until the diffusate was no longer radioactive (third change). At this point the sac contents accounted for 0.9×10^5 c.p.m., close to the expected value of 0.7×10^5 c.p.m. This material was freeze-dried and the residue was gel-filtered as described above for chymotrypsin treated with the same inhibitor.

Determination of the operational molarity of cathepsin B solutions. The concentration of cathepsin B used in the present study was determined by titrating the enzyme with an accurately determined amount of the active-site-directed inhibitor D-Phe-Phe-Arg-CH_2Cl (Shaw & Kettner, 1981).

Synthesis of peptidylfluoromethanes

Phthaloylphenylalanyldiazomethane (IIa). To phthaloylphenylalanine (6.5 g, 22 mmol) in tetrahydrofuran (110 ml) was added triethylamine (3.22 ml, 23.1 mmol) and isobutyl chloroformate (3.02 ml, 23.1 mmol) at −20 °C. After 15 min diazomethane (66 mmol) in diethyl ether was added. The reaction mixture was first stirred for 1 h at −20 °C, then for 1 h at room temperature. After the addition of water (100 ml) the reaction mixture was extracted with ethyl acetate; the organic phase was washed successively with satd. $NaHCO_3$ and satd. NaCl and then dried over $MgSO_4$, filtered and evaporated. The resulting yellow oily solid was recrystallized from ethyl acetate/hexane, giving light-yellow prisms (5.1 g, 73% yield), m.p. 142–144 °C (Found: C, 67.85; H, 4.22; N, 12.86; $C_{18}H_{13}N_3O_3$ requires C, 67.70; H, 4.10; N, 13.16%). They had i.r. (KBr) 2085, 1708 and 1645 cm^{-1}, and n.m.r. δ (p.p.m.) ([$^2H_6$]dimethyl sulphoxide) 3.22–3.55 (2H, m, CH_2), 5.06–5.18 (1H, m, CH), 6.58 (1H, s, CHN_2), 7.05–7.2 (5H, s, C_6H_5) and 7.83 (4H, s, phthaloyl).

Phthaloylalanyldiazomethane (IIb). The same procedure as for compound (IIa) was used to give a yellow oil (76% yield). It had i.r. (neat) 2110 cm^{-1}, and n.m.r. δ (p.p.m.) ([$^2H_3$] chloroform) 1.70 (3H, d, J = 7 Hz, CH_3), 4.92 (1H, q, J = 7 Hz, CH_3CH), 5.40 (1H, s, CHN_2) and 7.70–7.95 (4H, m, phthaloyl).

Phthaloylphenylalanylfluoromethane (IIIa). A solution of 52% HF in pyridine (107.7 ml) was prepared by slow addition of pyridine (27.7 ml) to 70% HF in pyridine (80 ml) at 0 °C. After 10 min phthaloylphenylalanyldiazomethane (IIa) (6.4 g, 20 mmol) was added and the reaction mixture was stirred for 1 h at 0 °C, then 18 h at room temperature. The reaction mixture was neutralized by adding satd. $NaHCO_3$ (600 ml) and solid $NaHCO_3$. The solution was extracted twice with ethyl acetate (500 ml), the combined extracts were washed successively with 2 M-HCl (350 ml) and satd. NaCl (200 ml) and then dried over $MgSO_4$, filtered and evaporated. The resulting oily solid was chromatographed over silica gel with chloroform/ethyl acetate (2:1, v/v), giving, after recrystallization from methylene chloride/hexane, colourless prisms (2.0 g, 33% yield), m.p. 135–137 °C (Found: C, 69.79; H, 4.79; F, 5.86; N, 4.53; $C_{18}H_{14}FNO_3$ requires C, 69.45; H, 4.54; F, 6.10; N, 4.50%). They had i.r. (KBr) 1668 cm^{-1}, and n.m.r. δ (p.p.m.) ([$^2H_6$]dimethyl sulphoxide) 3.16–3.48 (2H, AB part of the ABX system, CH_2), 5.28–5.40 (1H, X part of the ABX system, CH), 5.36 (2H, d of the AB system, J_{HF} = 45 Hz, CH_2F), 7.04–7.24 (5H, s, C_6H_5) and 7.84 (4H, s, phthaloyl).

Phthaloylalanylfluoromethane (IIIb). The same procedure as for compound (IIIa) was used, to give colourless prisms (5% yield), m.p. 107–108 °C (Found: C, 61.32; H, 4.28; F, 7.77; N, 5.83; $C_{12}H_{10}FNO_3$ requires C, 61.28; H, 4.29; F, 8.08; N, 5.96%). They had i.r. (KBr) 1780, 1755, 1725 and 1705 cm^{-1}, and n.m.r. δ (p.p.m.) ([$^2H_3$]chloroform) 1.70 (3H, d, J = 7 Hz, CH_3), 5.00 (2H, d, J_{HF} = 45 Hz, CH_2F), 5.10 (1H, m, $CHCH_3$) and 7.70–7.95 (4H, m, phthaloyl).

3-Amino-1-fluoro-4-phenylbutan-2-ol (IVa). To phthaloylphenylalanylfluoromethane (IIIa) (656 mg, 2.1 mmol) suspended in propan-1-ol/water (7:3, v/v) (21 ml) was added $NaBH_4$ (398 mg, 10.5 mmol); solution was obtained after 10 min of stirring. After stirring for 16 h at room temperature, h.p.l.c. showed complete consumption of starting material. Acetic acid (2.2 ml) was added and the reaction mixture was heated at 80 °C for 7.5 h. The reaction mixture was then evaporated, and the residue was dissolved in water, basified with 2 M-NaOH to pH 10 and extracted with ethyl acetate. The organic phase was treated with water, and the aqueous phase was acidified to pH 1 with 1 M-HCl, separated, washed with ethyl acetate and then freeze-dried to give a colourless clear oil (390 mg, 85% yield).

3-(*N*-Benzyloxycarbonylamido)-1-fluoro-4-phenylbutan-2-ol (Va). To a solution of 3-amino-1-fluorophenylbutan-2-ol (IVa) (390 mg, 1.78 mmol) and $NaHCO_3$ (596 mg, 7.10 mmol) in water (18 ml) was added, under ice/water cooling, a 50% (v/v) benzyl chloroformate solution in toluene (894 μl, 2.66 mmol). After 30 min toluene (4 ml) was added and the cooling bath taken away. H.p.l.c. showed no starting material left after 16 h. Ethyl acetate (50 ml) was added and the combined organic phases were washed three times with 1 M-$KHSO_4$ (50 ml, pH 2.2), water (10 ml) and satd. NaCl (10 ml). The organic phase was dried over $MgSO_4$ and was filtered and then evaporated to give an oily solid (509 mg, 90% yield), which upon crystallization from ethyl acetate/hexane (1:10, v/v) gave colourless needles

(238 mg, 47% yield) as a mixture (30:70) of the two diastereomers.

A 150 mg portion of the needles was recrystallized twice from ethyl acetate/hexane (1:5, v/v), yielding pure isomer (11 mg) as colourless needles, m.p. 154–156 °C (Found: C, 68.05; H, 6.35; F, 5.98; N, 4.52; $C_{18}H_{20}FNO_3$ requires C, 68.12; H, 6.35; F, 5.99; N, 4.41%). They had i.r. (KBr) 3320, 1695 and 1540 cm^{-1}, and n.m.r. δ (p.p.m.) ([$^2H_3$]chloroform) 2.94 (2H, d, $J = 7$ Hz, $C_6H_5CH_2CH$), 3.84–4.10 (2H, m, 2C*H*), 4.49 (2H, d of the AB system, $J_{HF} = 47$ Hz, CH_2F), 4.80–4.90 (1H, m, O*H*), 5.04 (2H, s, $C_6H_5CH_2O$) and 7.16–7.40 (11H, M, N*H*+ $2C_6H_5$).

The mother liquors were chromatographed on a preparative silica-gel plate with ethyl acetate/hexane (1:1 v/v), yielding after recrystallization from ethyl acetate/hexane colourless needles (25 mg) enriched in the other isomer (78:22), m.p. 75–78 °C (Found: C, 68.02; H, 6.50; N, 4.71; F, 6.05; $C_{18}H_{20}FNO_3$ requires C, 68.12; H, 6.35; F, 5.99; N, 4.41%). They had i.r. (KBr) 3360, 1675 and 1538 cm^{-1}, and n.m.r. δ (p.p.m.) ([$^2H_3$]chloroform) 2.96 (2H, d, $J = 7$ Hz, $C_6H_5CH_2CH$), 3.82–3.98 (2H, m, 2C*H*), 4.27 (2H, d of the AB system, $J_{HF} = 47$ Hz, CH_2F), 5.06 (3H, m, $C_6H_5CH_2O$ + O*H*) and 7.16–7.42 (11H, m, N*H*+$2C_6H_5$).

3-(*N*-t-Butoxycarbonylamido)-1-fluoro-4-phenylbutan-2-ol (VIIa). 3-Amino-1-fluoro-4-phenylbutan-2-ol (IVa) was treated with di-t-butyl dicarbonate to give, after chromatography on silica in chloroform, colourless needles (88% yield, 67:33 mixture of diastereomers), m.p. 90–100 °C (Found: C, 63.32; H, 7.94; F, 6.38; N, 4.55; $C_{15}H_{22}FNO_3$ requires C, 63.59; H, 7.83; F, 6.71; N, 4.95%). They had i.r. (KBr) 3430, 3300, 1685 and 1550 cm^{-1}, and n.m.r. δ (p.p.m.) ([$^2H_6$]dimethyl sulphoxide) 1.32 [9H, s, $(CH_3)_3C$], 2.58–2.90 (2H, m, $C_6H_5CH_2O$], 3.60–3.80 [2H, m, 2C*H*), 4.10–4.56 (2H, m, CH_2F), 5.27 (1H, d, $J = 5$ Hz, O*H*), 6.57 (1H, d, $J = 10$ Hz, N*H*) and 7.10–7.35 (5*H*, m, C_6H_5).

3-(*N*-Benzyloxycarbonylphenylalanylamido)-1-fluoro-3-phenylbutan-2-ol (IXa). Isobutyl chloroformate (82.3 μl, 0.63 mmol) was added to benzyloxycarbonylphenylalanine (210 mg, 0.7 mmol) and *N*-methylmorpholine (69.3 μl, 0.63 mmol) in tetrahydrofuran (5 ml) at −20 °C. After 10 min 3-amino-1-fluoro-4-phenylbutan-2-ol (IVa) (139 mg, 0.63 mmol) and *N*-methylmorpholine (69.3 μl, 0.63 mmol) in tetrahydrofuran (5 ml) were added. The reaction mixture was stirred for 1 h at −20 °C and then for 1 h at room temperature. Ethyl acetate and water were added, the phases were separated, and the organic phase was washed successively with satd. $NaHCO_3$ and satd. NaCl, dried over $MgSO_4$, filtered and evaporated. The resulting colourless solid was recrystallized from methanol/ethanol to give colourless needles (94 mg, 32% yield, 62:38 mixture of diastereomers), m.p. 150–160 °C. They had i.r. (KBr) 3300, 1750, 1690, 1660 and 1580 cm^{-1}, and n.m.r. δ (p.p.m.) [$^2H_6$]dimethyl sulphoxide) 2.60–3.08 (4H, m, $2C_6H_5CH_2CH$), 3.60–4.32 (5H, m, 3C*H*+ CH_2F), 4.96 (2H, s, $C_6H_5CH_2O$), 5.41 (38%) and 5.54 (62%) (1H, 2d, $J = 6$ Hz, O*H*), 7.10–7.40 (15H, m, $3C_6H_5$), 7.42 (38%) and 7.48 (62%) (1H, 2d, $J = 9$ Hz, N*H*) and 7.78 (62%) and 7.91 (38%) (1H, 2d, $J = 9$ Hz, N*H*).

3-(*N*-Benzyloxycarbonylphenylalanylamido)-1-fluorobutan-2-ol (IXb). Deprotection of phthaloylalanylfluoromethane (IIIb) followed by coupling with benzyloxycarbonylphenylalanine gave a colourless solid (71% yield, 60:40 mixture of diastereomers), m.p. 116–121 °C. It has i.r. (KBr) 3400, 1690, 1650 and 1530 cm^{-1}, and n.m.r. δ (p.p.m.) ([$^2H_6$]dimethyl sulphoxide) 1.04 (3H, d, $J = 7$ Hz, CH_3), 2.66–3.02 (2H, m, $C_6H_5CH_2$), 3.52–4.36 (5H, m, 3C*H*+CH_2F), 4.94 (2H, s, $C_6H_5CH_2O$), 5.24 (40%) and 5.34 (60%) (1H, d, $J = 7$ Hz, O*H*), 7.14–7.40 (10H, m, $2C_6H_5$), 7.50 (1H, d, $J = 9$ Hz, N*H*) and 7.72 (60%) and 7.91 (40%) (1H, d, $J = 9$ Hz, N*H*).

Benzyloxycarbonylphenylalanylfluoromethane (VIa). To a solution of CrO_3 (1.28 g, 12.8 mmol) and pyridine (2.06 ml, 25.6 mmol) in methylene chloride (32 ml) was added the alcohol (Va) (509 mg, 1.6 mmol, mixture of hydroxy isomers) in methylene chloride (5 ml). The reaction mixture was stirred for 16 h, and a further solution of CrO_3 (0.64 g, 6.4 mmol) and pyridine (1 ml, 12.8 mmol) in methylene chloride (16 ml) was added. After 45 min the reaction mixture was filtered through a short silica-gel column with ethyl acetate, and the solution was washed successively with 1 M-HCl and satd. NaCl, then dried over $MgSO_4$, filtered and evaporated. The resulting oil, which partly solidified, was purified by chromatography through a silica-gel column with chloroform. Recrystallization from ethyl acetate/hexane gave colourless needles (103 mg, 20% yield), m.p. 93–94 °C (Found: C, 68.55; H, 5.99; F, 5.82; N, 4.53; $C_{18}H_{18}FNO_3$ requires C, 68.56; H, 5.76; F, 6.03; N, 4.44%). They had i.r. (KBr) 3310, 1750 and 1692 cm^{-1}, n.m.r. δ (p.p.m.) ([$^2H_6$]dimethyl sulphoxide) 2.92 (2H, AB part of the ABX system, $C_6H_5CH_2CH$), 4.36–4.50 (1H, X part of the ABX system, $C_6H_5CH_2CH$], 4.98 (2H, s, $C_6H_5CH_2O$), 5.22 (2H, d of the AB system, $J_{HF} = 46$ Hz, CH_2F), 7.20–7.40 (10H, m, $2C_6H_5$) and 7.82 (1H, d, $J = 9$ Hz, N*H*), and $[\alpha]_D$ (chloroform) $+11.4 \pm 1.0°$.

t-Butoxycarbonylphenylalanylfluoromethane (VIIIa). Pyridine-SO_3 adduct (159 mg, 1 mmol) was added to the alcohol (VIIa) (89 mg, 0.31 mmol) and triethylamine (418 μl, 3 mmol) in dimethyl sulphoxide (1.5 ml). After the mixture had been stirred for 19 h at room temperature ethyl acetate was added. The solution was washed with water, dilute $KHSO_4$ (pH 2.6) until the aqueous phase was pH 2–3, satd. $NaHCO_3$ and satd. NaCl, and then dried over $MgSO_4$, filtered and evaporated. Purification on a silica-gel column with chloroform/methylene chloride (1:1, v/v) gave, after recrystallization from methylene chloride/hexane, colourless needles (44 mg, 50% yield), m.p. 99–101 °C (Found: C, 63.93; H, 7.18; F, 6.88; N, 4.73; $C_{15}H_{20}FNO_3$ requires C, 64.04; H, 7.17; F, 6.76; N, 4.98%). They had i.r. (KBr) 3360, 1750, 1695 and 1510 cm^{-1}, and n.m.r. δ (p.p.m.) ([$^2H_6$]dimethyl sulphoxide) 1.25, 1.35 [9H, 2s, $(CH_3)_3C$], 2.65–2.82, 2.95–3.10 (2H, AB part of the ABX system, $C_6H_5CH_2CH$), 4.24–4.38 (1H, X part of the ABX system, $C_6H_5CH_2CH$), 5.18 (2H, d of the AB system, $J_{HF} = 46$ Hz, CH_2F) and 7.15–7.40 (6H, m, N*H*+C_6H_5).

Benzyloxycarbonylphenylalanylphenylalanylfluoromethane (Xa). Pyridine–SO_3 adduct (44 mg, 0.27 mmol) in dimethyl sulphoxide (0.2 ml) was added to the alcohol (IXa) (31 mg, 0.067 mmol) and triethylamine (150 μl, 1.072 mmol) in dimethyl sulphoxide (0.4 ml). After 19 h ethyl acetate was added. The reaction mixture was washed successively with water, 1 M-HCl, satd. $NaHCO_3$ and satd. NaCl and then dried over $MgSO_4$, filtered and evaporated to give a brownish solid (22 mg). Purification on a silica-gel column with chloroform/methanol (97:3, v/v) and recrystallization from methylene chloride/hexane gave the colourless solid (Xa) as a racemate, m.p. 150–160 °C (Found: C, 70.29; H, 6.15; F, 4.09; N, 6.40; $C_{27}H_{27}FN_2O_4$ requires C, 70.11; H, 5.88; F, 4.11; N, 6.06%). It had i.r. (KBr) 3300, 1750, 1690, 1660 and 1580 cm^{-1}, and n.m.r. δ (p.p.m.) ([$^2H_6$]dimethyl sulphoxide) 2.55–3.20 (4H, m, $2C_6H_5CH_2CH$), 4.08–4.28 (1H, m, $C_6H_5CH_2CH$), 4.46–4.68 (1H, m, $C_6H_5CH_2CH$], 4.95 (2H, s, $C_6H_5CH_2O$), 5.15 (2H, d of the AB system, $J_{HF}=47$ Hz, CH_2F), 7.05–7.30 (15H, m, $3C_6H_5$) 7.46, 7.58 (1H, 2d, $J = 7$ Hz, NH, racemate), 8.59 and 8.64 (1H, 2d, $J = 7$ Hz, NH, racemate).

Benzyloxycarbonylphenylalanylalanylfluoromethane (Xb). The same procedure as for compound (Xa) was used to give colourless needles (26% yield, 60:40 mixture of diastereomers), m.p. 137–140 °C (Found: C, 65.19; H, 6.04; F, 4.91; N, 7.36; $C_{21}H_{23}FN_2O_4$ requires C, 65.27; H, 6.00; N, 7.25; F, 4.92%). They had i.r. (KBr) 3300, 1745, 1690, 1645 and 1535 cm^{-1}, n.m.r. δ (p.p.m.) ([$^2H_6$]dimethyl sulphoxide) 1.14 (60%) and 1.21 (40%) (3H, 2d, $J = 7$ Hz, CH_3), 2.70–3.04 (2H, m, $C_6H_5CH_2CH$), 4.18–4.40 (2H, m, 2CH), 4.95 (1H, 2s, $C_6H_5CH_2O$), 5.27 (2H, d of the AB system, $J = 45$ Hz, CH_2F), 7.13–7.40 (10H, m, $2C_6H_5$), 7.60 (40%) and 7.63 (60%) (1H, 2d, $J = 7$ Hz, NH) and 8.48 (60%) and 8.53 (40%) (1H, 2d, $J = 7$ Hz, NH), and $[\alpha]_D$ (chloroform) $-0.7 \pm 1.1°$.

[^{3}H]Acetylphenylalanylfluoromethane. The procedure used was an adaption to the micro scale of previous work on the conversion of t-butoxycarbonyl-Phe-CH_2F into the acetyl derivative, which was obtained analytically pure. t-Butoxycarbonyl-Phe-CH_2F (3.9 mg) was left in 1 ml of about 2 M-HCl in ethyl acetate at 0 °C for about 30 min, after which the solvent was removed in a stream of N_2. The residue in water (0.2 ml) was treated at 0 °C with a solution of [^{3}H]acetic anhydride (0.1 mmol, 5 mCi) in tetrahydrofuran (0.4 ml) and solid $NaHCO_3$ (11 mg) with intermittent shaking during 2 h. The reaction mixture was then allowed to warm up to room temperature for about 1 h and extracted with ethyl acetate. After being dried with $MgSO_4$, the solution was evaporated to dryness and the crystalline residue desiccated in high vacuum. The transformations were monitored by h.p.l.c. and the observed changes in retention time corresponded to those of the larger-scale model reaction. The residue was dissolved in ethanol to provide a 10 mM solution.

Stereospecific aspect of the fluoromethane synthesis

During the synthesis of the fluoromethanes racemization can occur [Cbz-Phe-Phe-CH_2F (Xa), Cbz-Phe-Ala-CH_2F (Xb)], but not necessarily [Cbz-Phe-CH_2F (VIa)], as indicated in the physical data of the oxidation products. The problem of the racemization was not further investigated in the present work.

RESULTS

The fluoromethyl ketones derived from the single amino acid, phenylalanine, were examined for their inhibitory effects on chymotrypsin. Of the series of derivatives described in the present paper, only these were considered likely to have inhibitory action on this enzyme. The dipeptide derivatives containing the Phe-Xaa sequence were expected to be substrates. Cbz-Phe-CH_2F was the major candidate for study and provided a progressive loss of activity of chymotrypsin with time that was clearly slower than that by the corresponding chloromethyl ketone. For example, Cbz-Phe-CH_2F (40 μM) gave a $t_{\frac{1}{2}}$ of about 150 min for the inactivation of chymotrypsin at 25 °C. At 37 °C this was reduced to 38 min. By contrast, Cbz-Phe-CH_2Cl (50 μM)

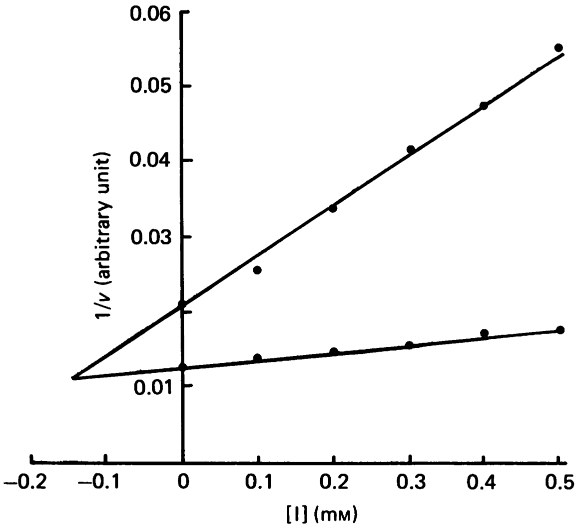

Fig. 1. Dixon plot of the inhibition of chymotrypsin by Cbz-Phe-CH_2F at 25 °C in 0.2 M-Tris/HCl buffer, pH 7.8, containing 5% ethanol

The substrate, Ala-Ala-Pro-Phe-*p*-nitroanilide (DelMar *et al.*, 1979), was either at 50 μM (upper line) or at 250 μM (lower line).

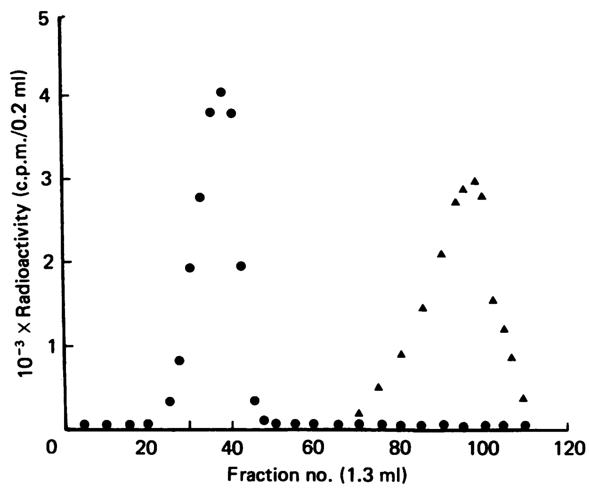

Fig. 2. Covalent modification of chymotrypsin by [^{3}H]acetyl-Phe-CH_2F

Gel filtration of the product in 6 M-guanidinium chloride on Sephadex G-75 (90 cm × 1.7 cm) shows radioactivity in the protein peak (●) well resolved from the position of free inhibitor (▲).

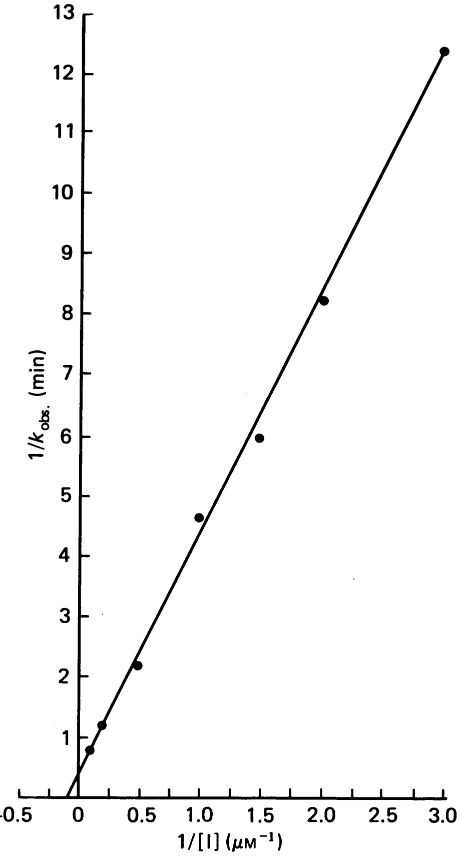

Fig. 3. Kitz–Wilson plot for the inactivation of cathepsin B by Cbz-Phe-Phe-CH_2F

The inhibition studies were carried out at 37 °C in 0.1 M-sodium acetate buffer, pH 5.4, containing 1 mM-EDTA and 0.02% 2-mercaptoethanol. The enzyme concentration was about 0.01 μM. The inhibitor concentration spanned the range 0.33–7.5 μM.

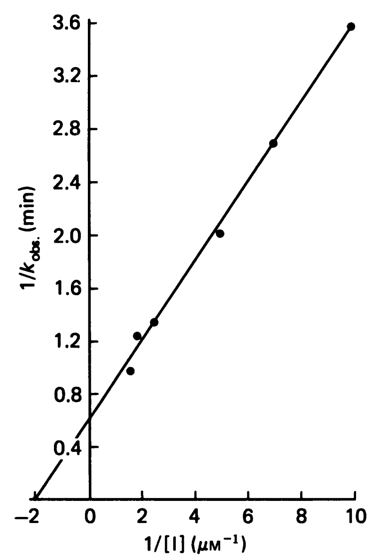

Fig. 4. Kitz–Wilson plot for the inactivation of cathepsin B by Cbz-Phe-Ala-CH_2F

Conditions were as indicated in Fig. 3 legend. The inhibitor concentration spanned the range of 0.1–0.55 μM.

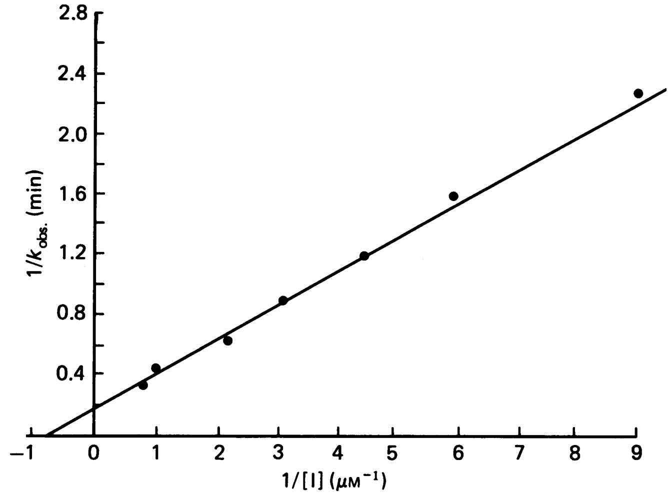

Fig. 5. Kitz–Wilson plot for the inactivation of cathepsin B by Cbz-Phe-Phe-CH_2Cl

Conditions were as indicated in Fig. 3 legend. The inhibitor concentration range was 0.1–1.25 μM.

gave a $t_{\frac{1}{2}}$ of about 2 min at 25 °C (Shaw & Ruscica, 1971). To relate this considerable difference in reactivity to differences in affinity or alkylation rate, an attempt was made to define the kinetic properties of Cbz-Phe-CH_2F by utilizing the method of Kitz & Wilson (1962). Reproducibility was poor, and such a comparative study was deferred. Because of the slow onset of irreversible action, it could also be readily studied as a reversible inhibitor by initial-velocity measurements made in the presence of succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (DelMar *et al.*, 1979). A K_i of 1.4×10^{-4} M was determined in this fashion by a Dixon plot (Fig. 1).

The progressive irreversible inactivation of chymotrypsin by the phenylalanylfluoromethanes is conceivably due to the relatively slow formation of a tight inhibitor–enzyme complex. On the other hand, a displacement reaction involving the active-centre histidine-57 would lead to the same alkylated enzyme formed by the chloromethanes known to reveal a loss of histidine on amino acid analysis due to failure of His-57 to be regenerated from the alkyl derivative on acid hydrolysis (Schoellmann & Shaw, 1963). In fact such a deletion was found in the case of inactivation by a fluoromethyl ketone, in which case the inhibited enzyme provided an arginine/histidine ratio of 3:1.2 compared with a ratio of 3:2 for uninhibited enzyme. Confirmatory evidence for covalent-bond formation was obtained in labelling experiments utilizing [^{3}H]acetyl-Phe-CH_2F. Chymotrypsin inactivated by this reagent retained its radioactivity after prolonged dialysis and also after gel filtration in 6 M-guanidinium chloride. The radioactivity was recovered in the protein peak (Fig. 2).

Similar studies were carried out on the effect of peptidylfluoromethanes on cathepsin B (EC 3.4.22.1) from pig liver, a proteinase with an essential cysteine thiol group. In this case the dipeptide derivatives containing a penultimate phenylalanine residue were eminently suitable, since this residue promotes affinity to the active centre of the proteinase, as shown by a variety of studies (Green & Shaw, 1981; Shaw & Kettner, 1981). Cbz-Phe-Phe-CH_2F inactivated cathepsin B in a manner

Table 1. Inactivation of cathepsin B by peptides containing *C*-terminal amino-acid-derived fluoromethanes and chloromethanes

All of the inactivation studies were carried out at 37 °C in 0.1 M-sodium acetate buffer, pH 5.4, containing 1 mM-EDTA and 0.02% 2-mercaptoethanol.

Inhibitor	K_i (M)	k_i (min^{-1})	k_i/K_i ($M^{-1} \cdot min^{-1}$)
Cbz-Phe-Phe-CH_2Cl	$2.3 \times 10^{-5} \pm 0.3 \times 10^{-5}$	12.5 ± 3	$5.4 \times 10^5 \pm 0.7 \times 10^5$
Cbz-Phe-Phe-CH_2F	$1.4 \times 10^{-5} \pm 0.3 \times 10^{-5}$	3.3 ± 0.6	$2.2 \times 10^5 \pm 0.5 \times 10^5$
Cbz-Phe-Ala-CH_2F	$5.5 \times 10^{-7} \pm 1.2 \times 10^{-7}$	1.8 ± 0.4	$3.2 \times 10^6 \pm 0.7 \times 10^6$

typical of affinity-labelling reagents, and the pseudo-first-order decay of enzyme activity at pH 5.4 as a function of inhibitor concentration provided a Kitz–Wilson plot indicative of formation of an intermediate complex (Fig. 3). As expected from results with the corresponding diazomethyl ketones (Watanabe *et al.*, 1979), Cbz-Phe-Ala-CH_2F was even more effective (Fig. 4 and Table 1), owing to tighter binding. For comparison, Cbz-Phe-Phe-CH_2Cl was also studied, and the results (Fig. 5 and Table 1) indicate that the reactivity of the peptidylfluoromethane is about half that of the corresponding peptidylchloromethane for cathepsin B if one compares the second-order rate constant for enzyme inactivation. The rate for covalent-bond formation is about one-fourth that of the chloromethane, but this is offset by tighter binding. As in the case of chymotrypsin, when the enzyme was inactivated with a radioactive form of a fluoromethane, the radioactivity was retained on gel filtration in 6 M-guanidinium chloride, appearing completely in the protein peak.

DISCUSSION

The observation that derivatives of phenylalanylfluoromethane alkylate the active-centre histidine residue of chymotrypsin is a striking demonstration of the ability of affinity labelling to evoke chemical reactivity. It appears that in the series Cbz-Phe-CH_2X the order of displaceability of fluoride will, at least in a qualitative sense, fall properly in a reactivity series containing other halides (Shaw & Ruscica, 1971) or departing groups susceptible to nucleophilic displacement (Larsen & Shaw, 1976). However, we do not yet have adequate data to compare the relative alkylation rates or affinities of analogous reagents, for example Cbz-Phe-CH_2Cl and the corresponding fluoro compound. The second-order rate constant for the inactivation of chymotrypsin by fluoromethyl ketone is about 1/40th that of the chloromethyl ketone at 25 °C at pH 7, i.e. 117 $M^{-1} \cdot min^{-1}$ compared with 4140 $M^{-1} \cdot min^{-1}$ (Shaw & Ruscica, 1971). Probably a large part of this difference is due to a lower rate for the covalent-bond-forming step. For Cbz-Phe-CH_2Cl such information is not readily available because of the high reactivity, limitations of conventional spectroscopic methods and the generally poor affinity of chymotrypsin for typical ligands (Kurachi *et al.*, 1973). A quantitative comparison of the kinetic properties of fluoromethyl ketones and chloromethyl ketones as inactivators of serine proteinases can more readily be achieved with members of the trypsin-like family, including plasmin and thrombin, which have more affinity for small ligands than has chymotrypsin.

On the other hand, the present work and the observations by Rasnick (1985) provide, in the case of a cysteine proteinase, a comparison of the kinetic properties of a chloromethyl ketone and a fluoromethyl ketone. The Cbz-Phe-Phe derivatives were used in our case at 37 °C, and the results (Table 1) are comparable with those reported by Rasnick (1985) obtained with the Cbz-Phe-Ala derivatives at 28 °C. The fluoromethyl ketones are about 35–40% as effective as the chloromethyl ketones if one compares the second-order rates of enzyme inactivation. A lowered rate of covalent-bond formation is the cause of this difference, as expected. In the case of the Cbz-Phe-Phe derivatives, the fluoromethyl ketone alkylation rate was 0.26 times that of the chloromethyl ketone at 37 °C, but in the overall reactivity this was offset by tighter binding in the reversible phase (Table 1). This pattern is qualitatively similar to the comparison of the Cbz-Phe-Ala derivatives examined by Rasnick (1985). We also studied Cbz-Phe-Ala-CH_2F at 37 °C, with results very similar to his; the differences can be attributed to the increased alkylation rate at the higher temperature used in our work. Finally, we observed that, when a radioactive fluoromethyl ketone was used to inactivate cathepsin B, the radioactivity was fully retained under denaturing conditions, and, as in the case of chymotrypsin (Fig. 2), appeared in the protein peak on gel filtration. Thus the inhibition is considered the result of modification of a typical affinity-labelling protein and not due to the formation of a tight-binding complex.

It is of interest that the rate of alkylation by fluoromethyl ketones compared with chloromethyl ketones is less depressed for the cysteine proteinase than for the serine proteinase by at least an order of magnitude.

In a study of the affinity labelling of triose-phosphate isomerase, Hartman (1971) showed that 1-halo-3-hydroxyacetone phosphates (iodo-, bromo- and chloro-) acting as analogues of the substrate dihydroxyacetone phosphate inactivated the enzyme irreversibly. Esterification of the active-centre glutamate side chain was shown to take place. In Fondy's laboratory, the fluoro analogue was obtained and was also found to inactivate triose-phosphate isomerase irreversibly, but the rate was about 1/1000th that of the chloro compound (Silverman *et al.*, 1975). Presumably esterification of the enzyme was taking place in this case also.

Although peptidylfluoromethanes are less reactive than the corresponding chloro compounds, nevertheless their affinities appear to be at least as good, and thus they provide inhibition of a reversible nature just as effectively as the chloromethyl ketones, although the onset of the irreversible phase is delayed. For use *in vivo* this may represent progress if diminished side reactions result in greater specificity and prolonged maintenance of inhibitor concentrations.

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